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(54) Title: PROCESS FOR PREPARING POLYUNSATURATED FATTY ACIDS

(57) Abstract

An advantageous process essentially based on the use of molecular distillation, for the preparation of polyunsaturated fatty acids, and namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and of their ethyl esters, from oils of animal and vegetable origin is disclosed, which is particularly suitable for large-scale industrial productions. By that way, complexes constituted by EPA and DHA, or by their ethyl esters, with a total concentration ranging from 35 up to 90 %, and products constituted by DHA alone, or by its ethyl ester at a total concentration not lower than 90 % can be obtained, and these mixtures are used for various purposes, ranging from dietetic-alimentary uses to typically pharmaceutical uses for the management of a very large number of alterations, malfunctions, diseases and pathologies.

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### Process for preparing polyunsaturated fatty acids

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The present invention relates to a process for preparing a high-concentration mixture of eicosapentaenoic acid and docosahexaenoic acid, and of their esters, by starting from oils of various animal and/or vegetable origins, as well as to the so obtained mixtures, and to their use for prophylactic or therapeutical purposes.

The process of the present invention is furthermore suitable for deodourising and deacidifying the same oils, in view of a possible dietetical or alimentary use thereof.

15 It is known by now that the polyunsaturated fatty acids

- 2 -

play an important role in human being physiology, because they perform, in particular, two roles; a structural role, as constituents of the phospholipids of the cellular membranes, and a functional role, as precursors of 5 prostaglandins.

The fatty acids belonging to the family of  $\alpha$ -linolenic acid perform in fact a basic task for the development and function of brain, retina and gonads, as well as for the formation of  $PGI_3$  and  $TxA_3$ , extremely important factors for 10 platelet agglutination preventive effect.

Among these, in particular, important are the long-chain members of  $\omega$ -3 family, i.e., eicosapentaenoic acid (20:5 $\omega$ -3), or EPA, and docosahexaenoic acid (22:6 $\omega$ -3), or DHA, which derive from the desaturation and chain extension 15 of  $\alpha$ -linolenic acid, thanks to the intervention of the relevant enzymes ( $\Delta$ -desaturases).

EPA, as a precursor of both  $PGI_3$  and  $TxA_3$ , performs an platelet agglutination prevention action and an antithrombotic effect which can be reconducted to an 20 inhibition of cyclooxygenase (an aspirin-like effect) and/or to the competition with arachidonic acid for this enzyme, with a consequent decreased synthesis of  $PGE_2$  and  $TxA_2$ , well-known platelet agglutinants.

DHA is the most important component of brain lipids in 25 man and is present at high concentrations in the

- 3 -

phospholipids of the synaptic membranes, a fact, this, which makes researchers suppose DHA to play a role in the transmission of nervous impulse.

Furthermore, inasmuch as it is a structural element of 5 platelet cell, DHA indirectly plays, by increasing platelet fluidity, an important role in antithrombotic action.

Recent studies on man evidenced a decrease of  $\Delta$ -6-desaturase enzyme with increasing age (after 35 years of age); as a consequence, an endogenous deficiency could occur 10 of above said acids, which therefore should be administered by means of the diet, or by means of suitable compositions. However, to date several practical difficulties have prevented a wide use of said acids to be made in therapy or 15 as alimentary integrators, a use which, on the other hand, would be highly desirable in view of the above reported biochemical and pharmacological background. Such difficulties are mainly related to the extraction of said acids from fish oils, their purification and concentration up to suitable values for a pharmaceutical use, and their 20 odourisation.

Although many methods have been proposed and published in the past, the above cited objectives have not been reached to a satisfactory extent yet, as, among others, the still now limited use of EPA and/or DHA demonstrates, 25 notwithstanding their considerable potentialities as drugs

- 4 -

or alimentary integrators. The methods known to date, based on different techniques, such as degreasing, counter-current extraction, urea addition, liquid chromatography, distillation, lead to rather low yields and to easily perishable products if exposed to light or to atmosphere. Furthermore, most known methods aim at purifying eicosapentaenoic acid only, to the detriment of other useful unsaturated fatty acids, such as DHA.

For example, U.S. patent 4,377,526 discloses a process for purifying EPA, or its esters, which comprises a treatment with urea, followed by fractional distillation. By such a method, percentages of EPA higher than 70% are obtained, whilst DHA remains present as a residue (3-5%) only.

Furthermore, as far as the present Applicant knows to date, all patented processes relating to the production of the same, or of similar, products, use a more or less complex combination of chemical or physical operations, such as, e.g., the use of urea for the preferential precipitation of less unsaturated acids (WO 87/03899, JP 57-187397), or extractions with supercritic fluids (JP 60-214757, IP 60-115698).

In order to reach high titers of DHA acids, or of esters thereof, in other patents also chromatography is used, with various chromatographic beds which range from

- 5 -

silica gel up to low-polarity copolymers (JP 61-291540, JP 61-037752, JP 58-109444, GB 2090529).

On the other hand, in those patents in which the molecular distillation is used (as, e.g., JP-113099), this 5 is not the characterizing step of the process, but is simply used as a means for a rough purification during the processing.

The process of the present invention is exclusively based, apart from the common hydrolysis of triglycerides in 10 order to obtain the acids, on the technique of molecular distillation. The molecular distillation is used by suitably changing the operating conditions, in order to obtain the whole range of products of the present invention, without any other chemical or physical treatments.

15 The object of the present invention is hence a method for extracting DHA and EPA ethyl esters and free fatty acids from raw oils of various kinds with high yields, under conditions easily applicable in the industrial field, and leading to a stable and odourless product, which can be used 20 in human therapy both as a pharmaceutical and as a dietetic and alimentary product.

Furthermore, such a process only requires a very small number of chemical treatments, in that it is substantially based on the articulated use of a technology, i.e., 25 molecular distillation, which, owing to its operating

- 6 -

conditions, secures the highest protection of the products of the invention. In fact, these products are known to be very subject to undergo phenomena of chemical and thermal degradation. Finally, from the standpoint of the industrial 5 economic feasibility, molecular distillation is per se suitable for a continuous production, with extremely low operating costs.

The only use of molecular distillation in the process of the present invention makes it possible the following to 10 be obtained:

- 1) high quality products, also because they are not submitted to a too large number of chemical processes;
- 2) products at even very high concentrations, which may reach, in case of EPA DHA mixture, a value of 90%, and, 15 in case of DHA alone, 96%; these concentrations are considerably higher than as claimed in corresponding prior patents;
- 3) products at different titers for different uses, ranging from dietetic-alimentary uses to the typically pharmaceutical use, by simply suitably varying the parameters of molecular distillation only;
- 20 4) the process according to the present invention is particularly suitable for an industrial production, contrarily to many of above cited patents, whose implementation from a laboratory level to the industrial 25

- 7 -

level is much more expensive and problematic;

5) a further advantage is that, in case the ethyl esters have to be obtained, such a preparation can be carried out as the last processing step, by converting the acid products, at a suitable concentration and titer, into the corresponding esters. This is a considerable advantage from the view point of the reduction of the industrial production costs, and furthermore provides a process which is not disclosed in any of prior patents;

10 6) the process of the present invention is furthermore ideal for an industrial production, because, if the necessary equipment is available, it can be carried out in continuous mode, with a minimum use of labour and at the highest production level.

15 The high titers (up to 90%) and the considerable degree of purity of the EPA/DHA mixtures obtained by means of the process according to the present invention make it possible the pharmaceutical effects to be better pointed out, which derive from the administration of poly-unsaturated acids of 20  $\omega$ 3 series, and, in particular, of EPA/DHA.

Having high concentrations of EPA/DHA, on one hand lower-weight, smaller-size pharmaceutical forms can be prepared, which are easier to ingest or administer, and, on the other hand, the number of daily intakes or 25 administrations can be reduced.

- 8 -

The typical characteristics of EPA/DHA products of the present invention make it hence possible a greater therapeutical and formulation advantage to be attained in hyperlipemiae and therewith correlated pathologies, in thromboses, in platelet agglutination, in cardiac infarction, in hypertension, as anticoagulants, in prevention of atherosclerosis, in cerebral infarction, in lesions and occlusions caused by vasomotor spasms, in diabetes and its complications, in acute and chronic inflammations, in self-immune syndromes, in preventing the side effects at gastroenteric level of non-steroid anti-inflammatory agents, in tumor prevention.

The ratio of EPA concentration to DHA concentration changes according to the natural contents of the organism from which both compounds are extracted (e.g., various fish species, fish oils, crustaceans, sea weeds, and so forth).

The therapeutical properties of mixtures prevailingly containing EPA/DHA, or of mixtures containing, besides other poly-unsaturated fatty acids, also EPA/DHA, have been described in the past in several patents, and in particular: in the treatment of thromboses, in hypercholesterolemiae, in myocardial ischemia (WO 87/03899), in the prevention of arteriosclerosis, in cerebral infarction, in hyperlipemiae, in cardiac infarction (EP-A1-0 228 314), in the prophylaxis 25 of atherosclerosis, as antithrombotic, as antihypertensive

- 9 -

(JP 62-091188), in thrombotic pathologies, in platelet agglutination, in self-immune syndromes, in acute and chronic inflammations, in atherosclerosis, cardiac infarction, in venous thromboses, in hyperlipemic states, in hypertension, in lesions and occlusions originated by vasomotor spasms, in diabetes (WO 87/02247), in the prevention of the side effects of non-steroid anti-inflammatory agents (EP-Al-0 195 570), in the prophylaxis and management of diabetes complications (JP 60-248610), in hypercholesterolemiae, in hypertriglyceridemiae (DE 34 38 630); as anticoagulants, in hypercholesterolemiae (BE 899 184). Furthermore, both EPA and DHA have an influence on the metabolism of poly-unsaturated fatty acids, promoting the formation of products endowed with a high biological activity, i.e., the ecosanoids, which are active in tumor prevention.

Such activities were evidenced by prevailingly using poly-unsaturated fatty acids of  $\omega$ 3 series, precursors of EPA and DHA (JP 57-187397 and BE 897 806).

The preparations, whose references have been hereinabove cited, are often true mixtures of poly-unsaturated fatty acids prevailingly belonging to  $\omega$ 3 series, and however, the EPA/DHA concentrations used are always considerably lower than those reached by means of the process according to the present invention.

- 10 -

DHA, a highly unsaturated, long-chain fatty acid, belongs to the series denominated as "W3". Differently from what occurs in lower animal species, wherein both eicosahexaenoic acid (EPA) and DHA are present, in man only 5 traces of EPA, and high concentrations of DHA are found.

DHA is present in exclusively esterified form in membrane glycerophospholipids, and, in particular, in some districts, such as the CNS, in synaptic membranes and in retinal cells.

10 To the poly-unsaturated fatty acids belonging to W3 series, and to EPA, metabolic precursors of DHA, an extremely high number of biological and therapeutical activities have been attributed.

15 In the metabolic pathway starting from  $\alpha$ -linolenic acid and leading to DHA, the administration of EPA does not lead, except for small amounts, to the conversion into DHA, whilst a portion of administered DHA is converted back into EPA.

In fact, the ingestion of DHA, in ester form, and/or as 20 the free acid, significantly increases both DHA and EPA levels in plasmatic phospholipids (Hiroi at al., 1978).

Thus, DHA, besides performing its own task, would also ensure, by being converted back into EPA, the biological actions typical of EPA.

25 In prior patents, several therapeutical activities have been claimed for mixtures of poly-unsaturated fatty acids of

- 11 -

WO series, to which DHA belongs, and, in particular, therapeutical activities have been claimed in hyperlipemiae and therewith correlated pathologies, in thromboses, in platelet agglutination, in cardiac infarction, in 5 hypertension, as anticoagulants, in atherosclerosis prevention, in cerebral infarction, in lesions and occlusions caused by vasomotor spasms, in diabetes and its complications, in acute and chronic inflammations, in self-immune syndromes, in preventing the side effects at the 10 gastroenteric level of non-steroid anti-inflammatory agents, and in tumor prevention (WO 87/03899, EP-A1-0 228 314, JP 62-091188, WO 87/02247, EP-A1-0 195 570, JP 60-248610, DE 34 38 630, BE 899 184, JP 57-187397, BE 897 806).

DHA, as a single substance, was evaluated in therapy as 15 a platelet agglutination preventive agent, and an use thereof in the prophylaxis of thrombotic processes was proposed (GB 2,098,065, GB-2,090,529).

In reality, the DHA used in the prior studies does not seem to have been as highly concentrated as DHA obtained by 20 means of the process according to the present invention.

Furthermore, the process of extraction of the present invention, by means of molecular distillation, without either chemical or physical treatments, characterizes the obtained DHA with a high purity degree, as compared to the 25 previously obtained products.

- 12 -

By means of different experimental models, the activity of highly concentrated (96%) and purified DHA in hyperlipemiae was pointed out. In fact, the administration of DHA reduced, to a meaningful extent, the experimentally induced high levels of cholesterol and triglycerides.

On considering the obtained results, on the basis of the functions which DHA performs inside the organism, and as a consequence of the phenomena observed in various districts when DHA is administered, its characteristics and therapeutic peculiarities can be summarized as follows: in the treatment and prophylaxis of dislipemic diseases and therewith connected pathologies, such as hyperlipoproteinemiae, hypercholesterolemiae, hypertriglyceridemiae, in the alterations of fat metabolism, in damages to vessels caused by cholesterol, in atherosclerosis, in xanthomas, in diabetic retinopathy, in the prevention of thrombus formation, in prevention of aortal and coronary arteriosclerosis, as a coadjuvant in those diseases which may originate manifestations of hyperlipoproteinemiae (diabetes mellitus, hypothyroidism, uraemia, and so forth), in cardiac infarction, in platelet agglutination, in hypertension, in anticoagulant therapy, in cerebral infarction, in acute and chronic inflammations, in diabetes, in self-immune syndromes, in the prevention of the side effects caused by non-steroid anti-inflammatory agents,

- 13 -

in tumor prevention, in retinopathies with visual deficit, in ceroidoses, in the processes relevant to learning and ageing.

The process according to the present invention is disclosed now in detail, and one will thus see that by means of said process, those purposes and advantages which have been hereinabove outlined can be fully achieved.

An alkaline hydrolysis (NaOH) of the raw oil is performed up to the complete breakdown of the triglycerides.

The solid soap formed is collected and is immediately acidified with mineral acid in an aqueous solution.

The formed acids are extracted with petroleum ether, up to exhaustion. The extracts are combined with one another, are thoroughly washed with water, and are concentrated up to total solvent removal.

The resulting product is processed by exclusively using molecular distillation, in such a way as to obtain the whole range of products according to the present invention.

A) COMPLEX CONSTITUTED BY EPA AND DHA

A - 1 Total concentration comprised within the range of from 35 to 40%

A - 2 Total concentration comprised within the range of from 40 to 50%

A - 3 Total concentration comprised within the range of from 50 to 60%

- 14 -

A - 4 Total concentration comprised within the range of from  
60 to 70%

A - 5 Total concentration comprised within the range of from  
70 to 80%

5 A - 6 Total concentration comprised within the range of from  
80 to 90%

B) PRODUCT CONSTITUTED BY DHA

B - 1 Total concentration 90%

B - 2 Total concentration 96%

10 - Process for obtaining A-1 product

The mixture of fatty acids obtained from the first step  
of the process (saponification) is submitted to molecular  
distillation, operating under a pressure of  $10^{-3}$  mm<sub>Hg</sub>, with  
a temperature of the evaporator of 110-120°C, in order to  
15 remove the process impurities and the natural impurities,  
which constitute the residue.

The distillate maintains the concentration of EPA and  
DHA which was originally present in the starting oil (15-20%  
of EPA and 15-20% of DHA, in case of a fish oil), and is  
20 free from above-said impurities; it constitutes, per se, the  
A-1 end product, and is the starting material for subsequent  
A-2, A-3, A-4 products.

- Process for obtaining A-2 product

25 A-1 product is submitted to molecular distillation,  
under a pressure of  $10^{-3}$  mm<sub>Hg</sub> and with an evaporator

- 15 -

temperature of 50°C. The residue is constituted by A-2 product, and the increase in EPA and DHA titer takes place to the detriment of the lower molecular-weight acids (C<sub>16</sub> and C<sub>18</sub>), which constitute the distillate.

5      - Process for obtaining A-3 product

A-1 product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is increased to 60°C. The residue is constituted by A-3 product, and the increase in EPA and DHA 10 titer takes place to the detriment of the lower molecular-weight acids (C<sub>16</sub> and C<sub>18</sub>), which constitute the distillate.

- Process for obtaining A-4 product

A-1 product is submitted to molecular distillation under the above-said conditions, except for evaporator 15 temperature, which is increased to 70°C. The residue is constituted by A-4 product, and the increase in EPA and DHA titer takes place to the detriment of the lower molecular-weight acids (C<sub>16</sub> and C<sub>18</sub>), which constitute the distillate.

- Process for obtaining A-5 product

20      A-4 product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is increased to 75°C. The residue is constituted by A-5 product, and the increase in EPA and DHA titer takes place to the detriment of the lower molecular-weight acids (C<sub>16</sub>-C<sub>18</sub> and lower-unsaturated-C<sub>20</sub>), which 25

- 16 -

constitute the distillate.

- Process for obtaining A-6 product

A-5 product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is of 80°C. The residue is constituted by 5 A-6 product, and the increase in EPA and DHA titer takes place to the detriment of the lower molecular-weight acids (C<sub>16</sub>-C<sub>18</sub> and lower-unsaturated-C<sub>20</sub>), which constitute the distillate.

10 - Process for obtaining B-1 product

A-6 product is submitted to a double molecular distillation under the above-said conditions, except for evaporator temperature, which is of 85°C. The residue is constituted by B-1 product (DHA 90%), and the distillate is 15 mainly constituted by EPA and minor amounts of other acids.

- Process for obtaining B-2 product

B-1 product is submitted to molecular distillation under the same condition as used for B-1, with the evaporator temperature being of 85°C. The residue is 20 constituted by 96% of DHA, and the distillate is mainly constituted by EPA and minor amounts of other acids.

The general process for producing the fatty acids ethyl esters can be alternatively based on the transesterification of the glycerides which constitute the original oil.

The transesterification process consists in treating the oils of various animal or vegetable origin with ethanol in an acidic medium according to the usual techniques.  
5

The reaction product is additioned to an equal volume of water and the whole mixture is extracted with petroleum ether or cyclohexane.

10 The organic extract is washed with water up to neutrality and is dried and concentrated until the complete solvent disappearance.

The obtained product is submitted to molecular distillation, operating under the successively disclosed conditions.

#### EXAMPLE OF PRACTICAL PROCESS

15 80 kgs. of fish oil and 50 kgs. of ethanol are placed in a mixing reactor in which have been previously dissolved 2.5 kgs of concentrated sulphuric acid.

The process is carried out in reflux conditions, with the reactor closed, under nitrogen atmosphere, taking the temperature up to  $82^{\circ}\pm 2^{\circ}\text{C}$ .

20 After 6 hours, we start to check if fatty acids glycerides are still present in the reagent mixture.

The control is realised by T.L.C. on silica gel plates using a mixture of petroleum ether: diethyl ether: acetic acid (85:14:1) as eluant.

The developer is constituted by a mixture of concentrated sulphuric acid and methyl alcohol 1:1.

25 The plates treated with this mixture are placed for few minutes at  $105^{\circ}\text{C}$ : the triglycerides become visible as dark spots near the starting line. When the

chromatographic control shows the end of the reaction, the heating circuit is stopped and the liquid mixture is distilled in order to remove the excess of ethanol.

The residue of the distillation is left to cool up to ambient temperature:  
5 then 200 kgs of water and 150 kgs of petroleum ether or cyclohexane are added.

These ones are shaked and the water discharged.

Water washing (portions of 200 kgs) is repeated 3-5 times, until neutral reaction of the discharge.

The cyclohexane solution is dried with anhydrous sodium sulphate and the  
10 cyclohexane is removed by vacuum distillation (20 mmHg) at 60°C.

The residue is stored under nitrogen atmosphere and is ready for the next stage of molecular distillation.

C) COMPLEX CONSTITUTED BY EPA AND DHA ETHYL ESTERS

C-1 Total concentration comprised within the range of from 35 to 40%

15 C-2 Total concentration comprised within the range of from 40 to 50%

C-3 Total concentration comprised within the range of from 50 to 60%

C-4 Total concentration comprised within the range of from 60 to 70%

C-5 Total concentration comprised within the range of from 70 to 80%

C-6 Total concentration comprised within the range of from 80 to 90%

20 D) COMPLEX CONSTITUTED BY DHA ETHYL ESTER

D-1 Total concentration 90%

D-2 Total concentration 90%

- Process for obtaining C-1 product

The mixture of fatty acid ethyl esters obtained from the above mentioned  
25 transesterification is submitted to molecular distillation, operating under a

- 19 -

pressure of  $10^{-3}$  mmHg, with a temperature of the evaporator of 90-110°C, in order to remove the process impurities and the natural impurities, which constitute the residue.

The distillate maintains the concentration of EPA and DHA which was originally present in the starting oil (15-20% of EPA and 15-20% of DHA, in case of a fish oil), and is free from above-said impurities; it constitutes, per se, the C-1 end product, and is the starting material for subsequent C-2, C-3, C-4 products.

Process for obtaining C-2 product

C-1 product is submitted to molecular distillation, under a pressure of  $10^{-3}$  mmHg and with an evaporator temperature of 50°C. The residue is constituted by C-2 product, and the increase in EPA and DHA ethyl ester titer takes place to the detriment of the lower molecular-weight acid ethyl esters ( $C_{16}$  and  $C_{18}$ ), which constitute the distillate.

Process for obtaining C-3 product

C-1 product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is increased to 60-70°C. The residue is constituted by C-3 product, and the increase in EPA and DHA ethyl esters takes place to the detriment of the lower molecular weight acid ethyl esters ( $C_{16}$  and  $C_{18}$ ), which constitute the distillate.

Process for obtaining C-4 product

C-1 product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is increased to 70-80°C. The residue is constituted by C-4 product, and the increase in EPA and DHA ethyl esters titer takes place to the detriment of the lower molecular weight

acid ethyl esters ( $C_{16}$  and  $C_{18}$ ), which constitute the distillate.

Process for obtaining C-5 product

C-4 product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is increased to 80-90°C.

5 The residue is constituted by C-5 product, and the increase in EPA and DHA ethyl esters titer takes place to the detriment of the lower molecular weight acid ethyl esters ( $C_{16}$  and  $C_{18}$  and lower-unsaturated- $C_{20}$ ), which constitute the distillate.

Process for obtaining C-6 product

10 A certain quantity of solvent (ethyl alcohol or acetone or similars) equal to about 8 times the quantity of C-5 to be treated, is added of a quantity of urea equal to approx. the double quantity of C-5.

The mixture is heated until the complete dissolution of the urea; to this one the foreseen quantity of C-5 is added.

15 After cooling, the precipitate that appears is filtered and the alcoholic solution is vacuum concentrated at a small volume.

Water is added and the whole mixture is extracted with an equal quantity of cyclohexane.

20 The organic phase is washed with water several times, to remove every trace of urea.

After all, the organic solution is dried and the solvent is totally removed by vacuum distillation.

This product is submitted to molecular distillation under the above-said conditions, except for evaporator temperature, which is of 70-90°C. The residue 25 is constituted by C-6 product, and the increase in EPA and DHA ethyl esters

- 21 -

titer takes place to the detriment of the lower molecular weight acids ethyl esters ( $C_{16}$  and  $C_{18}$  and lower-unsaturated- $C_{20}$ ), which constitute the distillate.

Process for obtaining D-1 product

5 C-6 product is submitted to a double molecular distillation under the above-said conditions, except for evaporator temperature, which is of 75°-95°C. The residue is constituted by D-1 product (DHA ethyl ester 90%), and the distillate is mainly constituted by EPA ethyl ester and minor amounts of other acid ethyl esters.

Process for obtaining D-2 product

10 C-1 product is submitted to molecular distillaiton under the same condition as used for D-1, with the evaporator temperature being of 75-95°C. The residue is constituted by 96° of DHA ethyl esters and the distillate is mainly constituted by EPA ethyl ester and minor amount of other acid ethyl esters.

15 The following examples are given in order to illustrate the process of the present invention in greater detail, and in no way they should be construed as being limitative of the scope of protection of the present invention.

Example no. 1

100 kg of raw oil is dissolved in 150 lts. of ethanol to 95° and added of 5 kg of concentrated sulphuric acid.

20 The whole mixture is place under  $N_2$  and in reflux as long as every trace of triglycerides is disappeared.

25 The greater part of the solvent is removed under vacuum. To the residue is added a quantity of water equal to 5 times the same residue and it is extracted with a convenient quantity of solvent (petroleum ether, or cyclohexane or similars). The organic extract are washed up to complete neutrality, dried and

- 22 -

concentrated under vacuum up to total removal of the solvent.

The product obtained in this way composed by a mixture of ethyl esters of the fatty acids constituting the triglycerides of the starting product, is loaded in the molecular still operating in one of the above-said conditions.

5

Example 2

The process for obtaining C-6 product consists in treating C-5 product with urea and, successively, in a molecular distillation.

120 lts of ethanol at 95° and 20 kgs of urea are heated up to complete dissolution of the urea. To this solution 15-20 lts of C-5 are added and the whole mixture is shaked, keeping the heating under Nitrogen.

10

After cooling it is filtered removing the precipitate.

15

The resulting alcoholic solution is concentrated under vacuum at a small volume. This oily residue is additioned of 80-100 lts of water and 80-100 lts of solvent (petroleum ether or cyclohexane or similar); this one is shaked, the organic part is extracted and washed with water several times. The organic phase is dried and the solvent is completely evaporated under vacuum.

This product is introduced in the molecular still and distilled in the above-said conditions to obtain a residue which is constituted by C-5 product.

20

As additional clarification, of the production process, the phases required to obtain the relative products are now described with reference to a plant specially designed for the implementation of the process itself.

The production process basically consists of three main phases plus certain intermediate and final treatments which are necessary for the success of the processing and the definitive purification of the product.

25

The processing involved can be schematically described as follows:

- 23 -

1. Transesterification of the original triglycerides in ethyl esters - identified by the initials ETR
2. First clarification treatment with earth - identified by the initials TTE I
3. Concentration by molecular distillation - identified by the initials DMV
- 5       4. Treatment with urea - identified by the initials TRU
- 5       5. Second clarification with earth - identified by the initials TTE2
6. Definitive removal of processing solvents - identified by the initials DF

It is clear that phases 1, 3, 4 are those which are determinant for obtaining the end product.

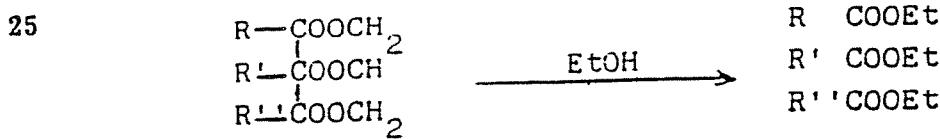
10     Analytical controls are carried out between each phase of the process to ensure correspondance with the set specifications.

1. TRANSESTERIFICATION: ETR

It is obviously more convenient for the subsequent phase of molecular distillation to have available ethyl esters of polyunsaturated fatty acids. As we 15 shall see later, by its very nature, molecular distillation is a technique which permits distillation at temperatures much lower than ordinary temperatures, temperatures at which there would be product degradation. Thus, working with ethyl esters, there is an additional increase in volatility compared to the triglycerides and, therefore, an additional safeguard for the delicate cis-20 polyunsaturated structure of the fatty acids in which we are interested.

In addition, transesterification permits deodorisation of the product, the loss of all eventual hydrosoluble impurities and the destruction of possible residues of vitamins A and D.

#### 1.1 Transesterification process (ETR)



- 24 -

where R, R', R'' are alkyl residues in the range C<sub>14</sub> - C<sub>24</sub> at various levels of unsaturation. Assuming an initial amount of 500 kg of fish oil, the following quantities of reagents must be used:

	100% denatured ethyl alcohol (2% MEC)	312.5 kg
5	Sulphuric acid	26.250 kg
	Deionised water	2500 kg
	Cyclohexane I	540 kg
	Cyclohexane II	250 kg
	Sodium sulphate	100 kg

10 Apparatus used (see diagram 1)

TA101 - TA106	INOX steel loading and temporary storage containers
RE 106	Enamel reactor of 3 m <sup>3</sup> with codant for reflux and for distillation
RE 107	Inox steel reactor of 3 m <sup>3</sup>
15 DSL 15	Mixing reactor of 3 m <sup>3</sup>
F 15	Inox steel filter

Practical process (whole process carried out under nitrogen atmosphere)

1. Oil, alcohol and sulphuric acid are loaded from TA101, 102, 103 into RE 106: time 6h.
- 20 2. Heating up to reflux: time 1h.
3. 10-12 hours of reflux. Sampling to check completion of reaction.
4. Vacuum distillation of ethanol: time 6h.
5. Cooling of mass and transfer to RE107, which already contains the deionised water and the first load of cyclohexane from TA 104: time 2 h.
- 25 6. Agitation of the two-phase for 1/2 h. Agitation stops for as long as is

- 25 -

required to obtain total separation of the two phases.

7. The lower aqueous phase is separated and transferred to TA 106.
8. The organic phase is transferred to TA 105.
9. The aqueous phase in TA 106 is returned to RE 107 and the second weight  
5 of cyclohexane is loaded from TA 104.
10. The process described in point 6 is repeated.
11. The lower aqueous phase is separated and eliminated by transfer to the  
purification plant.
12. The organic phase is transferred to DSL 15. Total time from point 5 to  
10 point 12: 8 - 10 h.
13. Anhydrous sodium sulphate is added to the solution in DSL 15 and then  
agitated for at least 3 h. The mixture is then filtered through F 15 under  
hydrogen atmosphere. The solution is transferred to RE 106. Time: 6 h.
14. Distillation and complete stripping of the solvent in a current of nitrogen.  
15 Time 7 - 8 h.
15. Transfer to a drum.

At the end of the process the mixture of ethyl esters presents itself as  
freely flowing liquid, dark brown in colour, with a chromatographic profile of the  
type shown in Appendix A.

20 2. CLARIFICATION: TTE 1

At the end of the ETR phase, we proceed with phase 2 which should be  
considered a logical continuation of phase 1.

In reality, this processing step was not introduced immediately into the  
phases of the process and it is still being verified today.

25 The presence of this first clarification stage became necessary in order to

totally eliminate the solid corpuscles but also, primarily, to eliminate substances of an epoxy and peroxidic (coloured) nature which can trigger off processes of deterioration during storage. But, even more important, these processes of deterioration can occur inside the molecular still (favoured here by the high 5 temperatures) leading to the formation of strata of polymers on the internal wall of the still. On the one hand, this interferes with the distillation process, altering the conduction of heat, and on the other hand, makes necessary frequent and expensive maintenance operations.

### 2.1 Practical process

10 1. The operation can be carried out directly on the cyclohexane solution referred to in point 13 of the ETR practical process, and in the same apparatus (see diagram 2).

An amount of Tonsil 70 CC FF earth equal to 15% of the theoretical yield in esters is weighed out.

15 2. The weighed quantity of earth is added to the cyclohexane solution.  
3. The solution is heated up to a temperature of 80° C for 30', constantly maintaining the agitation and the flow of hydrogen.  
4. After cooling, it is filtered through F 15. The end product is transferred to INOX steel collection tanks where it is kept under hydrogen overpressure.  
20 5. As point 14 and 15 of the preceding ETR practical process.

Total time of phases ETR and TTE 1: 54 hours.

The efficiency of the filtration can be increased by the addition of a further passage of the solution through a filter press.

25 The mixture of ethyl esters is then collected in suitable INOX steel containers under nitrogen overpressure and 0.03% of tocopheral is added. The

- 27 -

yield of the whole process is 100%.

Tests

The required analytical tests are carried out on the esters obtained in this way:

5        - gas-chromatograph titre: in accordance with raw material specification.  
          - peroxidic number: lower than 10.  
          - residual solvents: lower than 250 ppm (G.C. app. 5)

The latter test is extremely important since a product with a quantity of solvent higher than the specification is not acceptable because it would interfere  
10      with the molecular distillation process.

**3. MOLECULAR DISTILLATION: DMV**

A brief description of molecular distillation will be given and then the various steps of the process will be described.

**3.1 Molecular distillation and short-path distillation**

15      Molecular distillation is a relatively young separation technique which has not yet been widely exploited at industrial level. It can solve numerous problems which cannot be solved by conventional distillation since:

20      - thermally unstable substances can be treated;  
          - high levels of purity can be obtained;  
          - there is a substantial reduction in energy consumption.

In addition, molecular distillation does not pollute and, in this specific case, it produces subproducts which can be totally utilised.

Molecular distillation is based on the following principle. A thin film (molecular) of the product to be distilled is spread over a heated wall and the molecules remain there for the time required to acquire the energy needed for

- 28 -

evaporation. Since this is done in conditions of high vacuum, the molecules have a free path and temperatures are kept well below normal boiling temperature. The volatile fractions are then collected on a cold wall.

The molecular distillation process operates in the following way:

5 the material to be distilled is fed into the top of the distillation column. As the material enters the column it is distributed over the heated wall of the still by rotating rollers.

Thus a liquid film is formed over the whole of the internal surface of the still and the more volatile molecules evaporate because of the operating 10 conditions (temperature, degree of vacuum). The evaporated molecules are then captured on a condenser.

The liquid film is continuously renewed by the rotating elements, ensuring that the substance does not remain on the heated wall for any length of time and, thus, avoiding the possibility of degradation. Any material which has not 15 evaporated flows down the wall as the result of gravity and is collected at the end as residue.

Short-path distillation represents a considerable advance in the technique of molecular distillation. Here, the distance between the heated wall and the condenser is considerably reduced and thus the molecule which has detached 20 itself from the heated wall encounters the cooling wall immediately and condenses on it. Therefore, the molecules do not collide on the brief path which they travel and, even more important, an extremely high and uniform vacuum can be obtained because of the immediate condensation of the molecules and thus the operating temperature can be further lowered providing an additional 25 safeguard for the stability of the product.

- 29 -

The diagram in fig. 1 illustrates the principle of short-path distillation.

Figure 2 shows a schematic of an evaporator of industrial dimensions while figure 3 shows a schematic drawing of a laboratory column, in which the parts in grey represent the so-called "wiper system" which forms a uniform film on the heated wall thus avoiding the formation of hot points which could damage the integrity of the product. The wiper system adopted in this column consists of a rotor with rods mounted vertically on the base. Hollow independent rollers made of glass fibre coated with teflon are mounted on these rods.

During rotation, these rollers are driven against the heated wall by centrifugal force and they form the film.

### 3.2 Description of the plant

This is a two-stage plant with the following characteristics:

<u>Quantity of product which can be processed</u>			
minimum	g/h	10 - 20	
maximum		3000	
<u>Heat exchange surfaces</u>			
evaporator	dm <sup>2</sup>	2 x 4.3	
condenser		2 x 2.2	
<u>Heating</u>			
energy required	Kw	2.7	
variable temperature		250	
<u>Rotation speed</u>			
	rpm	25 - 2000	
<u>Working pressure</u>			
with and without diffusion pump	mbar	1.10 <sup>-3</sup> /5.10 <sup>-2</sup>	
25 <u>Dimensions</u>			

- 30 -

length	mm	1200
width		400

Diagram 3 shows the primitive version of the molecular distillation plant as originally designed as an experimental pilot plant, while diagram 4 shows the 5 current modified and improved configuration.

The main modifications were the introduction of a preliminary degassing stage (4) and a metering pump (5) which permitted an increase in the hourly flow compared to the first version and also permitted independent supply for a certain number of hours. Finally, graduated cylinders (8) provided with venting valves 10 and relative automatic pumping system (10), permitting the removal of the product without interrupting production.

It is obvious that these modifications improve the productivity of the whole plant, bringing it closer to that of an industrial production plant.

### 3.2.1 Maintenance operations

15 The modified plant does not require particularly numerous and complex maintenance operations, and those which are required can be divided into three categories:

- a) check of the oil levels in the rotary and diffusion pumps.
  - Oil change for the rotary pump after 1000 working hours or after visual 20 inspection of the oil leads to the conclusion that it has deteriorated and needs to be changed.
  - Oil change for the diffusion pump every 500 working hours or when, because of accidents, the plant has lost vacuum to the extent that a considerable quantity of oxygen has come into contact with the oil.
- 25 - Check of the oil level and periodic change of the oil in the heater

- 31 -

thermostats.

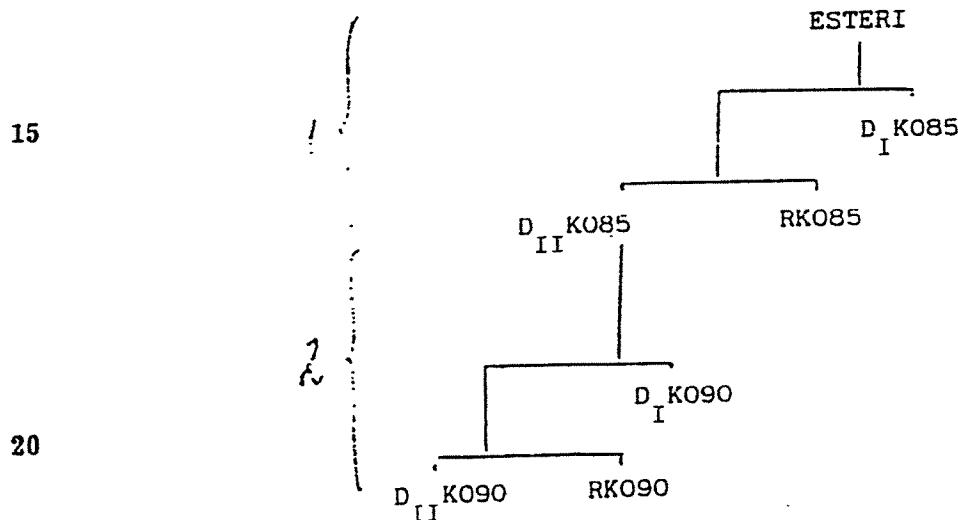
- b) weekly dismounting and cleaning of the heads of the metering pump. Check of the oil level.
- c) weekly cleaning of the distillation column.

5        - periodic replacement of the reflen rollers constituting the wiper system.

### 3.3 Description of the process

The molecular distillation is carried out on the derivate of the transesterification process previously described.

The EPA - DHA enrichment subsequent to molecular distillation is  
10 achieved in two passages through the two - stage distillation unit. The process can be schematically represented in the following way.



Schematic 5: The initials shown above are used in the description below.

At the end of the first passage a distillate indicated with the initials D<sub>II</sub>  
25 K085 is obtained with an average content of 45% of EPA + DHA.

- 32 -

The product collected at the end of the second passage R2 is indicated by the initials RK090 and has an average content of 58% of EPA + DHA.

**Process for obtaining D<sub>II</sub> K085 (see diagram 2)**

The initial esters are loaded into the dosing vessel (3) and then are pumped 5 by the metering pump (1) to the preliminary degassing stage (4), maintained at a temperature of 60° C and under a vacuum of around 5, 10<sup>-2</sup> mbar.

The flow is at a constant value of 1350 ml/h.

The esters pass from the degasser to the first evaporation column under the following operating conditions: temperature 115° C, vacuum 1.10<sup>-3</sup> mbar, 10 wiper system speed 350 rpm.

The residue of the first evaporation passes into the second column through the running trap (11).

The operating conditions are: T 125° C, vacuum 1.10<sup>-3</sup> mbar, wiper system speed 300 rpm. The temperature of the condenser in both evaporators is kept at 15 a constant level of 8° C.

The distillate is collected in suitable containers under nitrogen atmosphere.

**Process for obtaining RK090**

D<sub>II</sub> K085 is loaded into the recipient 3.

Metering pump flow (2): 1250 ml/h.

20 Conditions of first evaporator: temperature 105° C, vacuum 10<sup>-3</sup> mbar, wiper system 350 rpm.

Conditions of the second evaporator: temperature 90° C, vacuum 10<sup>-3</sup> mba, wiper system 350 rpm.

**Summarised table of the D.M. operating parameters**

- 33 -

		Stage I	Stage II	Stage I	Stage II
	Degasser T (°C)	60	60	60	60
	Condenser T(°C)	8	8	8	8
	Evaporation column (°C)	115	125	105	90
5	Degasser Pressure (mbar)	$5 \cdot 10^{-2}$	$5 \cdot 10^{-2}$	$5 \cdot 10^{-2}$	$5 \cdot 10^{-2}$
	Pressure in the evaporator column (m/bar)	$10^{-3}$	$10^{-3}$	$10^{-3}$	$10^{-3}$
	Flow (ml/h)	1350		1250	
	Rotation speed (rpm)	350	300	350	350

10        It must be remembered that the operator can introduce minimal variations into the process parameters indicated above from time to time in order to optimise production. In fact, since this is a natural product, wide variations in the composition are possible. It must also be remembered that there is a certain variability in the flow produced by the metering pump. This variability is very 15 probably due to two factors:

- 1)        the two basic components of the pump metering mechanism are two ball valves. Because of the nature of the material which is treated, these can become partially blocked and can cause the inconvenience described above in spite of the fact that maintenance is duly carried out.
- 20        2)        The viscosity of the product can vary slightly and this can be reflected in the variations in the flow.

However, this variability in the flow should not exceed the fixed value by more than 2-3%, otherwise there could be large variations in the compositions of the products.

25        The residue indicated by the initials RK090 is the end product deriving

- 34 -

from the molecular distillation. It is collected in suitable stainless steel containers after the addition of 0.03% of tocopheral under overpressure nitrogen atmosphere.

It is clear in colour, varying from a deep yellow to a pale orange yellow.

5 A sample is taken and subjected to the following routine tests:

- gas chromatography titration
- peroxidic index.

#### **Yield from molecular distillation**

400 kg of D<sub>II</sub> KO85 and 200 kg of RK 090 can be obtained from 1000 kg of  
10 ethyl esters, in other words an overall yield of 20%.

Returning to diagram 3, we can see how the intermediate products can be reutilised.

- RK085 is a product with a high content of DHA, varying between 50 and 60% and is the base for obtaining a concentrate of DHA. RK085 represents  
15 16% of the initial product.
- D<sub>I</sub> K085 is a product with a low content of EPA/DHA and, therefore, it cannot be reutilised in any stage of the process. However, this product is of considerable industrial interest since it can be reutilised in the cosmetics industry and the paint industry.
- 20 D<sub>I</sub> K085 constitutes about 48% of the initial esters.
- D<sub>I</sub> K090 and D<sub>II</sub> K090 both have an interesting average content of EPA and DHA, with a clear preponderance of EPA (attached chromatograms H, I). They represent about 16% of the total.

Some TRU tests were carried out on pooled distillates D<sub>I</sub> K090 and D<sub>II</sub>  
25 K090.

- 35 -

These tests showed that it was possible to recover part of these products and that, thus, they could be reinserted in the production cycle, naturally increasing the final yield in this way.

#### 4. TREATMENT WITH UREA: TRU

##### 5 4.1 Introduction

The concentrated product RK090 coming from the molecular distillation process is chemically treated with urea. This operation permits a minimum titre of 85% to be obtained leaving the EPA/DHA ratio practically unchanged.

And the latter factor was the basic reason which made the chemical treatment necessary. A titre of 85% can also be obtained by physical means through molecular distillation but this has negative consequences on the final yield and the EPA/DHA ratio of the product is firmly tilted in favour of the DHA. (Product of the type EPD 30%, DHA 55%).

This is not in line with the fixed objectives for two reasons:

- 15 1) in the present stage, general interest in fish oil is mainly due to its EPA content and, therefore, presentation of a product with the characteristics described above would be less successful.
- 2) the product of this process must reflect as faithfully as is possible the natural EPA/DHA ratio of the sardine.

20 The action of the urea forms an adduct, whose nature is not known, preferentially with fatty acid ethyl esters with low levels of unsaturation. This adduct is heat soluble and precipitates in a compact mass when the solution is gradually cooled.

The solid adduct can then be filtered to separate the solid adduct from the 25 solution containing high titre polyunsaturated fatty acid ethyl esters.

- 36 -

#### 4.2 Practical process.

For an initial amount of 9.1 Kg of RK090 (about 10 litres) the following quantities of reagents must be used:

- 64 Kg of alcohol 96% v/v (about 80 litres)
- 5 15 Kg of technical urea
- 354 Kg of 15% sodium chloride solution
- 31.6 Kg of cyclohexane

The whole process is conducted under nitrogen atmosphere.

The ethyl alcohol and the weighted amount of urea are placed in a reactor 10 equipped with a vertical scraper agitator. The internal temperature is brought up to 70°C until the urea is completely dissolved.

At this point the solution is cooled slightly (around 66°C) and the RK090 is added by means of the feeding device. When all the product has been added, the temperature is brought up to 70°C again and kept at that temperature for 10 15 minutes, the inflow of vapour stops and gradual cooling to ambient temperature (20°C) takes place. The reaction mixture is left in these conditions for 12 hours.

Cooling to an internal temperature of 20°C and, at the same time, leaving the reaction mixture alone for at least 12 hours are essential conditions for the success of the reaction.

20 At this point, the mixture is filtered.

The contents of the first reactor are discharged and pressure filtered directly on to a panel of diatomaceous earth. Pressure is exerted on the filter by a flow of nitrogen which does not exceed 1.5 Atm.

25 The clear solution is transferred to another reactor where it undergoes concentration under vacuum.

During the concentration stage there is further precipitation of excess urea which has not reacted with the ethyl esters.

This is what prevents the total elimination of the alcohol and in fact a doughy mass is formed which would become solid and unmixable if there were  
5 further distillation of ethanol.

The residue of the concentration is recovered with a first portion of 15% NaCl (55 litres) and extracted with cyclohexane. After this first extraction, the organic phase is washed three times with 15% NaCl solution. Washing is complete when the wash water gives a negative reaction when tested for  
10 presence of urea. Nessler's reagent can be used to carry out this test.

The extraction stage is particularly long and delicate because of the fact that emulsions are formed which are difficult to resolve.

A frothy portion forms at the interface of the two phases in which a certain quantity of the product is present. To help the resolution of emulsion, it  
15 can be heated up to 40-50°C. At the same time the aforesaid intermediate portion can be filtered and subsequently re-extracted.

However, a suitable organic solvent which does not permit the formation of emulsions can also be used.

The cyclohexane solution is anhydrated by means of the addition of  
20 anhydrous sodium sulphate. The clear solution is conveyed to a second reactor where it undergoes final decolouring.

This operation is a repetition of the operation already described in point 2.1. In this case the percentage of Tonsil 70 CC FF earth is different, equal to 10% in weight of the theoretical yield of the finished product.

25 5. FINAL PURIFICATION: DF

This consists of a further rapid passage of the finished product through the molecular still in order to remove definitively the residual solvents of the TRU treatment.

The finished product is analysed by gas chromatography showing a total  
5 EPA+DHA titre of not less than 85% and an EPA/DHA ratio of EPA 40-40%,  
DHA 40-46%.

DHA acts, through several mechanisms, on some metabolic  
10 processes and body districts, favouring a precise pharmacological placing thereof in some pathologies. It is important that preparations of DHA are used, which contain at least 5-10% of alpha-tocopherol, in order to prevent phenomena of peroxidation to the detriment of DHA, which is  
15 easily subject to oxidative processes, owing to the high unsaturation level thereof.

1) Action of DHA on metabolism of poly-unsaturated fatty acids.

In nature, several families of fatty acids exist, to each  
20 of which compounds belong, which are correlated with one another from a metabolic standpoint.

The three main families of poly-unsaturated acids are constituted by those compounds which belong to n-9, n-6 and n-3 metabolic series. By means of each of said short names, fatty acids are meant, which are endowed with the  
25

- 39 -

characteristic of respectively having the nearest double bond to methyl end (and simultaneously most far away from carboxy end) at a distance of 9, 6 and 3 C atoms.

Inasmuch as the fatty acid molecule portion comprised between the methyl end and the nearest double bond to it is not modified during the metabolic transformations (desaturation and chain extension) which the molecule can undergo, it derives that all compounds formed by metabolic interconversion maintain unchanged this structural characteristic. The respective parent compounds of the mentioned metabolic series of fatty acids are oleic acid, linoleic acid and alpha-linoleic acid, to which DHA belongs.

Linoleic acid and alpha-linoleic acid cannot be synthetized in upper organisms.

Such compounds, which play important biological roles in upper organisms, are hence essential compounds from a nutritional viewpoint, and must be intaken by means of food.

The reactions of metabolic conversion of the above indicated fatty acids into longer-chain, higher-unsaturated compounds take place by means of the activity of desaturation enzymes (desaturases) and chain-extending enzymes (elongases), prevailingly located at liver level (reticuloendothelial system).

The following considerations are important, as relates to the metabolism of the various series of poly-unsaturated fatty acids.

a) Regulation steps for such reactions exist, and, in

5 particular, the desaturation reactions are limiting steps.

b) The speeds of conversion of the precursors of the three series of fatty acids are very different from one another; such a speed is much higher for alpha-linoleic acid 18:3 (n-3 series), much lower for linoleic acid (18:2, n-6), and minimum for oleic acid.

c) A competitive antagonism exists in the metabolism of the three series of poly-unsaturated fatty acids, with the consequent inhibition, by the larger-affinity acids, of the metabolic conversion of the lower-affinity acids. On the contrary, the lack in the diet of acids with a high affinity for the enzymatic systems (e.g., the lack in essential linoleic and alpha-linoleic acids) unblocks the conversion of lower-affinity acids (oleic acid).

Therefore, in case of deficiencies of essential fatty acids, a conversion of oleic acid up to eicosatrienoic acid, C 20:3 n-9 takes place.

d) As a consequence of the metabolic interactions between the various unsaturated fatty acids (n-3, n-6, n-9)

- 41 -

supplied with the diet, it happens that the mutual ratio thereof conditions the metabolic conversion of C<sub>18</sub>-compounds belonging to the various series into more-unsaturated, longer-chain compounds, and, consequently, their incorporation with plasmatic and tissular lipids.

The administration of poly-unsaturated acids of n-3 series, such as DHA, is hence very important as to their metabolic use, and their incorporation with the tissues. In fact, it is evident that the administration, e.g., of DHA, will cause an incorporation of such compound with the cellular lipids, which will depend on several factors, such as the relative levels of n-6 acids in the diet, besides the relative affinity of DHA, as compared to that of n-6 acids, for the various phospholipidic pools in different cellular types.

2) Action of DHA on vascular district, on atherosclerosis, platelet functions and thrombus formation.

As a consequence of what reported above on the influence of DHA on the metabolism of unsaturated fatty acids, the role is important which it may play on C<sub>20</sub> poly-unsaturated fatty acids, precursors of products endowed with a high biological activity, viz., eicosanoids.

Eicosanoids are substances prevailingly deriving by enzymatic oxygenation from arachidonic acid (AA), through

the following two main routes: cyclooxygenase, leading to the formation of prostaglandins, prostacycline and thromboxane, and lipoxygenase, leading to the formation of hydroxyacids and leukotrienes.

5 Some specific eicosanoids may take an action in the mechanisms which regulate important functions, and which constitute the basis for some processes, such as in the formation of thrombi, and in vascular district (activation of production of  $I_3$  prostacycline, or  $PGI_3$ , instead of  $PGI_2$ ).

10 Through the formation of the specific eicosanoids and the consequent production of inactive  $TxA_3$ , DHA also acts on platelet agglutination (Rao G.H.R. et al., Biochem. Biophys. Res. Comm., 117, 549, 1983). However, the 15 effects on some systems of the administration of purified DHA are rather different from the effects exhibited by EPA.

In fact, although after DHA administration a platelet 20 agglutination preventive activity takes place, no particular interferences with the metabolism of AA were observed, differently from what occurs when EPA is administered (Hirai et al., in "Advances in Prostaglandins, Thromboxane and Leukotriene Research", Vol. 17, Eds. Samuelsson B., Paoletti R. and Ramwell 25 P.W., Raven Press, N.Y., page 838, 1978). Such data

suggests that DHA incorporated with platelet lipids is neither easily released, nor, consequently, converted into other compounds.

Furthermore, DHA, according to the studies by Talesnik and Carleton (Talesnik J, and Carleton Hsia J., Eur. J. Pharmacol., 80, 255, 1982), inhibits, at a coronary level, the vasoconstriction induced by AA.

It derives therefrom that DHA, through direct conversion, or reconversion into EPA with intervention on the metabolism of C<sub>20</sub>-fatty acids, can lead to the formation of specific eicosanoids, with the possibility of acting, at a preventive level, or at a therapeutical level, as an antithrombotic, in extending the bleeding times, as a coronary vasodilator, and as a platelet agglutination inhibitor.

3) Action of DHA on immunity system and in inflammation

The fatty acids belonging to n-3 series, to which DHA belongs, play an important role in varying the immunity and inflammatory responses, through the modifications in the cellular poly-unsaturated fatty acids, with consequent changes in the synthesis of prostaglandins and leukotrienes, in the cells engaged in the immunity and inflammatory responses (leukocytes, monocytes, T and B lymphocytes).

DHA performs an action in the inflammatory process on the

synthesis of prostaglandins through the competitive inhibition of the conversion of arachidonate into PGE<sub>2</sub>, as it occurs for some non-steroid anti-inflammatory agents, such as indomethacin (Corey E.J. et al., Proc.

5 Natl. Acad. Sci., 80, 3581, 1983).

Furthermore, various other processes of cellular activation can be modified by changes in the poly-unsaturated fatty acids in structural membrane lipids, as a consequence of the administration of fatty acids of n-3 series. Among these, an important role has to be assigned to the mobilization of intracellular Ca after stimulation, and to the generation of inositol phosphates from membrane phosphoinositide pools.

10 4) Action of DHA on visual functionality, ceroidosis, in learning and ageing processes

15 High concentrations of DHA are contained in retina and in synaptic terminations.

The depletion of DHA in such structures, obtained by means of dietetic manipulations in laboratory animals, causes visual malfunctions. (Tinoco J. et al., Biochim. Biophys. Acta, 486, 575, 1977).

20 Therefore, the availability of highly purified DHA in the treatment of those pathologies correlated with a decrease in DHA concentrations in retinal glycerophospholipids seems to be highly interesting from a therapeutical

25

viewpoint.

DHA depletion causes alterations in behaviour, with learning deficit. In fact, as Lamptey and Walker were able to observe (Lamptey M.S. and Walker B.L., J. Nutr., 106, 86, 1976) the learning capability shown by rats submitted to diets with different added amounts of n-3 acids was directly proportional to cerebral levels of 22:6 (DHA). Particularly interesting is also the correlation between ceroidosis and DHA concentration.

10 Ceroidosis is a pathology wherein a lipofuscinic pigment of brown colour (ceroid) can deposit inside cells of smooth-muscles of digestive tube, in liver, in muscles and in CNS.

15 Pullarkat et coworkers (Pullarkat R.K. et al., Neuropädiatrie, 9, 127, 1978) evidenced that in juvenile neuronal ceroidosis a correlation exists between the decrease in leukocytic DHA, found in all examined patients, and the seriousness of the disease. Furthermore, the same Authors observed, in a prior study, a decrease in DHA content in phosphatidylserine present in cerebral gray in adult and juvenile forms of neuronal ceroidosis.

20 25 The supply of exogenous, highly-purified DHA may be regarded as a valuable therapeutical means in the evolution of such a pathology.

As reported in the foreword, DHA is found in upper animals in ester form in membrane glycerophospholipids. Among main tissular glycerophospholipids (phosphatidyl-choline, PC or lecithin; phosphatidyl-ethanolamine, PE; phosphatidylserine, PS; and phosphatidyl-inositol, PI), and, in particular, PE and PS at a cerebral level, contain relatively high levels of DHA. The relative concentrations of DHA, relatively to total fatty acids, at the level of membranes of preparations of synaptosomes may reach values as high as 20%, with a ratio of >1 relatively to AA, the main poly-unsaturated acid present in other tissues. Said two phospholipids, PE and PS, are localized on the inner cellular membrane surface, and therefore the high DHA levels in such phospholipids suggest that such a fatty acid may be involved in some functions inside the cell. It is also interesting to observe that PC and PI, at the cerebral level, either contain very low DHA levels, the first one, or do not contain any DHA at all, the second one (Galli C. et al., in "Advances in Prostaglandins and Thromboxane Research", vol. 4, Eds. Coceani F. and Olley P.M., Raven Press, N.Y., page 181, 1978).

In the same work, the particular abundance of 22:6 (DHA) in phospholipids of synaptosomal membranes is observed. The administration of a diet containing an oil rich in n-

- 47 -

3 acids, and in particular of DHA, such as a fish oil, causes a sharp increase of this poly-unsaturated acid in membrane lipids.

5 Also in man (White H.R. et al., J. Neurochem. 18, 1337, 1971) decrease at cerebral level during the course of ageing.

In lipids isolated from cerebral synaptosomes of aged rats, a significant decrease in DHA is observed as compared to the values which are observed in adult rats. 10 Studies on rat also demonstrated that the capability of incorporating DHA administered by means of the diet, with cerebral lipids, considerably decreases during ageing (Eddy D.E., Harman D., J. Am. Ger. Soc. 25, 220, 1977). These Authors postulated that such a decrease is due to 15 an increase in lipid peroxidation at the cerebral level during the course of ageing.

Therefore, the possibility of being able to use concentrated DHA in all those complex mechanisms which lead to the learning and ageing process seems to be of 20 particular moment.

Furthermore, of considerable interest appears to be the fact that several proprietary medicines used in medical practice contain cerebral phospholipids, the natural seat of DHA, with the following operating mechanisms and 25 indications.

5 Phosphatidylserine: influence on the parameters of cerebral metabolism, altered during ageing. Therapeutical applications in chronic cerebral psycho-organic syndromes and valuable use in therewith correlated symptoms (lack in memory - confusion - poor attention and concentration, emotional lability, irritability, depressed mood, anxiety).

10 Diencephalic phospholipids: The liposomes of hypotalamus phospholipids are capable of activating the hypotalamus metabolism, increasing dopamine turnover, the activity of tyrosine-hydroxylase and of adenylycyclase, with a consequent increase in cyclic AMP. This effect reflects itself in particular on the functionality of hypotalamus-hypophysis axis. Application as a coadjuvant in cerebral metabolic alterations consequent to neuroendocrine disorders such as depressive syndromes, anxious-depressive statuses occurring during developmental age, in climacteric syndrome and in hypoprolactinemiae.

15

20 Cerebral phospholipids: are capable of activating the neuronal metabolism, normalizing the enzymatic activities of membranes, increasing the neurotransmitters turnover. Therapeutical application in neurologic syndromes such as arteriosclerotic cerebrovascular pathologies, involution syndromes, parkinsonian syndromes, crano-encephalic traumatic lesions and psychosomatic hypoevolutism,

25

- 49 -

as well as in all pathologies connected with altered statuses of CNS metabolism.

5) Action of DHA on cardiac functionality

The interest in the favourable effects of n-3 acids on various biologic parameters above all of cardiovascular district arouse nearly ten years ago.

10 The key observations which played a determining role in promoting this interest were some epidemiological studies carried out on populations (in particular the Eskimos) consuming very large amounts of fish, very rich in DHA, which evidenced an extremely low incidence of cardiovascular pathologies, notwithstanding the high contents of fats in their diet.

15 On rat, it was observed that the metabolism and the function at the cardiac level, in animals submitted to diets with different compositions as for fatty acids, are correlated with the contents of DHA in cardiac phospholipids (Guðbjarnason S. et al., Acta Biol. Med. Germ., vol. 37, 777, 1978).

20 In fact, the exposure to catecholamine-stress involved the replacement of linoleic acid by DHA in cardiac lipids, whilst cardiac frequency in groups of animals submitted to different diets was proportional to the DHA contents of the same lipidic fractions.

25 The therapeutical properties and prevention capabilities

of DHA, in certain cardiac diseases, are probably also bound, besides a direct action, to the different activities thereof in such different compartments as anti-atherosclerotic activity, antithrombotic activity, 5 hypolipemic activity and platelet agglutination preventive activity.

6) Action of DHA on hyperlipemiae

Hyperlipoproteinemic pathology is a condition wherein the concentration of cholesterol or of the lipoprotein-bearing triglycerides in plasma exceeds the normal physiologic limits.

At present, the percentage of population affected by high values of plasmatic concentration of cholesterol and/or triglycerides is estimated to be around 95% of total.

These limits vary according to age and sex.

Hyperlipoproteinemiae can be subdivided into primary and secondary hyperlipoproteinemiae.

Primary hyperlipoproteinemiae may be subdivided into two main groups: the monogenic primary hyperlipoproteinemiae (of genetic origin) and the polygenic primary hyperlipoproteinemiae (probably in already predisposed individuals, with the addition of incorrect diets and obesity).

The secondary hyperlipoproteinemiae evidence themselves 25 as complications of metabolic disturbances in some

- 51 -

pathologies, as diabetes mellitus, hypothyroidism, uremia, overdrinking, or as secondary effects in the use of oral contraceptives in subjects genetically prone to hypertriglyceridemia.

5 It is ascertained by now on scientific grounds that high concentrations of lipoproteins accelerate the development of arteriosclerosis, which may subsequently cause such irreparable damages as thrombosis and cardiac infarction. According to computations, in the U.S.A. approximately  
10 half deceases are likely to be due to such events.

With the evolution of industrialized civilization and with the relevant increase in social welfare, people, especially over past decades, started to overindulge in food, both as regards the amounts, and, from the dietetic-nutritional viewpoint, the quality.  
15

In fact, the intake hase progressively increased of food rich in cholesterol and saturated fatty acids. This is one of the main causes of the increase in dislipemic pathologies.

20 The first therapeutical aid for all hyperlipoproteinemiae is the use of a diet which maintains a normal body weight and reduces the concentrations of lipids in blood.

The elimination from the diet of saturated animal oils, to be replaced by poly-unsaturated vegetable oils, is a  
25 priority condition for lipidic illnesses to show a

- 52 -

positive evolution.

Due to this reason, we took into consideration the possibility that DHA, a poly-unsaturated long-chain fatty acid, may perform an action in dislipemic pathology.

5 Therefore, some preliminary pharmacologic tests were developed, in order to evidence the possible activity of DHA, by oral way, in test animals.

In all tests, Sprague-Dawley albino rats were used.

Esterified highly-concentrated DHA, having a titer of 10 96%, was used.

A group of animals per each test were treated with clofibrate or nicotinic acid, well-known molecules endowed with hypolipemic activity, in order to verify the experimental validity.

15 All animals, at sacrifice time, were under fasting conditions.

A) Activity of DHA in hyperlipemia induced by Nath diet.

Nath diet is a diet above all rich in cholesterol and cholic acid and, when intaken for a 4-5 weeks time, causes hyperlipemia in the animal.

20 Male rats of an initial weight of approximately 80-100 grams were used.

The animals were subdivided into 6 experimental groups:

1) controls (C);

25 2) controls + Nath diet (CD);

- 53 -

- 3) clofibrate, 300 mg/kg daily + Nath diet (CL);
- 4) DHA, 50 mg/kg daily + Nath diet;
- 5) DHA, 150 mg/kg daily + Nath diet;
- 6) DHA, 500 mg/kg daily + Nath diet.

5       The test lasted 5 weeks, during which the general conditions of the animals (growth, food consumption, etc.) were monitored.

10      At test end, the animals were sacrificed under fasting conditions, in order to evaluate the parameters (total cholesterol, triglycerides)

The results obtained confirm that the administrations of highly concentrated DHA inhibit the hyperlipemic effects experimentally induced by the nourishing with the Nath diet (Figure 4).

15      B) Activity of DHA on hyperlipemia induced by Triton WR 1339.

Triton WR 1339 or Tyloxapol is a substance capable of increasing the hematic levels of triglycerides and cholesterol in the laboratory animal.

20      Male rats of approximately 200 g of initial weight were used.

The animals were subdivided into 6 experimental groups:

- 1) controls (C);
- 2) controls + Triton (CT);

- 3) nicotinic acid, 500 mg/kg daily + Triton (AN);
- 4) DHA, 50 mg/kg daily + Triton;
- 5) DHA, 150 mg/kg daily + Triton;
- 6) DHA, 500 mg/kg daily + Triton.

5 The animals, before receiving Triton WR 1339, were pre-treated for a 2-days period with nicotinic acid, DHA or with the carrier only.

10 Eighteen hours after the treatment with Triton WR 1339, the animals, under fasting conditions, were sacrificed for the evaluation of the hematic levels of total cholesterol and triglycerides.

15 In this test too, the administration of DHA evidenced capability of this compound of decreasing the levels of the plasmatic lipids altered by the treatment with Triton WR 1339 (Figure 5).

C) Activity of DHA in hypertriglyceridemia induced by ethanol

The subacute administration of ethanol causes, in rat, a condition of hypertriglyceridemia.

20 For this test, male rats of an initial weight of approximately 200 grams were used.

The animal were subdivided into six test groups:

- 1) controls (C);
- 2) controls + ethanol (CE);
- 25 3) clofibrate, 200 mg/kg daily + ethanol (CL);

- 4) DHA, 50 mg/kg daily + ethanol;
- 5) DHA, 150 mg/kg daily + ethanol;
- 6) DHA, 500 mg/kg daily + ethanol.

Oral administrations of ethanol ad libitum in solution  
5 at 10%, alternating with administrations of 0.5 g/rat,  
induce hypertriglyceridemia in rat.

Since the day prior to the first intake of ethanol,  
the animals were treated with the substances under  
test. On the 4th day, 2 hours after the last treatment  
10 with ethanol, the animals were sacrificed in order to  
determine the triglycerides.

In this test, very reassuring results were obtained.  
In fact, the oral administration of high-concentration  
DHA significantly inhibited hypertriglyceridemia  
15 induced by ethanol (Figure 6).

Summing up, on considering the results obtained in the  
experimental tests, the use of high-titer DHA (96%) in the  
therapy of hyperlipemic pathologies and in the therewith  
correlated pathologies can be regarded as at all justified.

20 Finally, it is to be pointed out that many modifications, variants,  
additions and/or replacements of elements, process steps and operating  
details can be supplied to the process of the present invention, without  
thereby departing from the spirit or from the purview of said invention,  
and without departing from the protecting scope thereof, as it is also  
defined in the hereto appended claims.

- 56 -

C l a i m s

1. Process for preparing high-concentrated mixtures of poly-unsaturated fatty acids and their esters, from oils of animal and/or vegetable origin, characterized in that the  
5 raw oil is submitted to an alkaline hydrolysis, the solid soap so formed is acidified with a mineral acid in aqueous solution, the resulting mixture is extracted with petroleum ether up to exhaustion and then, after washing and concentration, the combined extracts are submitted to one or  
10 more molecular distillation step(s), with the pressure and temperature parameters being suitably changed, in order to obtain the whole range of the desired end products.

2. Process according to claim 1, characterized in that the first molecular distillation is carried out under a  
15 pressure of  $10^{-3}$  mmHg, and at a temperature of the evaporator of approximately 110-120°C.

3. Process according to claim 1, characterized in that the subsequent molecular distillation steps are carried out at an increasing temperature of from 50 to approximately  
20 80°C, in order to increase the titer of the poly-unsaturated fatty acids obtained, to the detriment of acids having progressively increasing molecular weight.

4. Process according to claim 1, characterized in that the poly-unsaturated fatty acids obtained are mainly  
25 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

-57-

5. Process according to claim 4, characterized in that in order to obtain a residue nearly exclusively constituted by DHA at a high concentration, higher than 90%, the products from the previous steps of molecular distillation 5 are submitted to a further molecular distillation at a higher temperature, not lower than approximately 85°C.

6. Process according to one or more of the preceding claims, characterized in that in order to prepare the ethyl esters of the poly-unsaturated fatty acids obtained from the 10 molecular distillation, the fatty acids are treated with ethanol in an acidic medium, to the reaction product an equal volume of water is added, and the whole mixture is extracted with petroleum ether, the ethereal solution, after being dried, is evaporated up to total removal of the 15 solvent, and the product is then submitted again to molecular distillation at a high temperature of approximately 100°C, in order that the process impurities are totally removed.

7. Mixture of poly-unsaturated fatty acids, whenever 20 obtained by means of the process according to one or more of claim(s) 1-5.

8. Mixture of ethyl esters of poly-unsaturated fatty acids, whenever obtained by means of the process according to claim 6.

25 9. Mixture according to claim 7, characterized in that

-58-

the poly-unsaturated fatty acids are mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

10. Mixture according to claim 9, characterized in that the total concentration of the mixture is comprised within 5 the range of from approximately 35 to approximately 90%, according to the number of steps of molecular distillation carried out.

11. Product essentially constituted by DHA at a total concentration of approximately 90%, whenever obtained by 10 means of the process according to claim 5.

12. Mixture of ethyl esters of EPA and DHA, whenever obtained by means of the process according to claims 1-7.

13. Product constituted by DHA ethyl ester at a high concentration, of at least 90%, whenever obtained by means 15 of the process according to claims 1-7.

14. Use of the products or mixtures according to claims 7-13 as dietetic-alimentary additives.

15. Use of the products or mixtures according to claims 7-3 as pharmacologically active substances, for prophylactic 20 or therapeutical purposes.

16. Use of mixtures according to claims 7-10 and 12 for the treatment and prevention of hyperlipemiae and therewith correlated pathologies, in thromboses, in platelet agglutination, in cardiac infarction, in hypertension, as 25 anticoagulants, in atherosclerosis prevention, in cerebral

- 59 -

infarction, in lesions and occlusions caused by vasomotor spasms, in diabetes and its complications, in acute and chronic inflammations, in self-immune syndromes, in preventing the side effects at gastroenteric level of non-  
5 steroid anti-inflammatory agents and in tumor prevention.

17. Use of DHA or of its ethyl ester according to claims 11 and 13 for the treatment and prevention of dislipemic diseases and therewith connected pathologies, such as hyperlipoproteinemiae, hypercholesterolemiae,  
10 hypertriglyceridemiae, alterations of fat metabolism, damages to vessels caused by cholesterol, atherosclerosis, xanthomas, diabetic retinopathy, in the prevention of thrombus formation, in prevention of aortal and coronary arteriosclerosis, as a coadjuvant in those diseases which  
15 may originate manifestations of hyperlipoproteinemiae (diabetes mellitus, hypothyroidism, uraemia, and so forth), in cardiac infarction, in platelet agglutination, in hypertension, in the anticoagulant therapy, in cerebral infarction, in acute and chronic inflammations, in diabetes,  
20 in self-immune syndromes, in the prevention of the side effects caused by non-steroid anti-inflammatories, in tumor prevention, in retinopathies with visual deficit, in ceroidoses, in the processes relevant to learning and ageing.

1/8

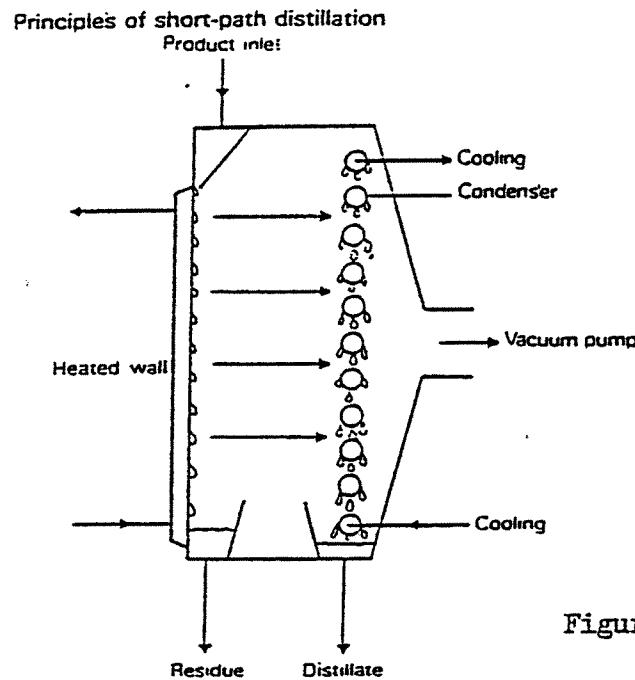


Figure 1

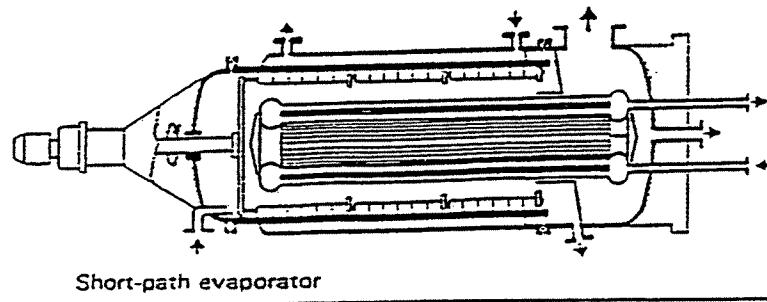


Figure 2

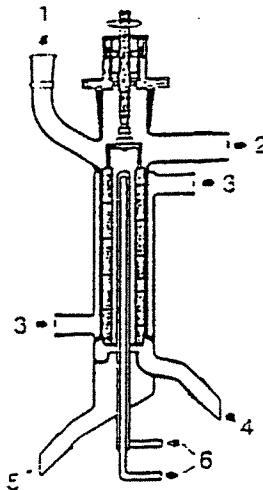
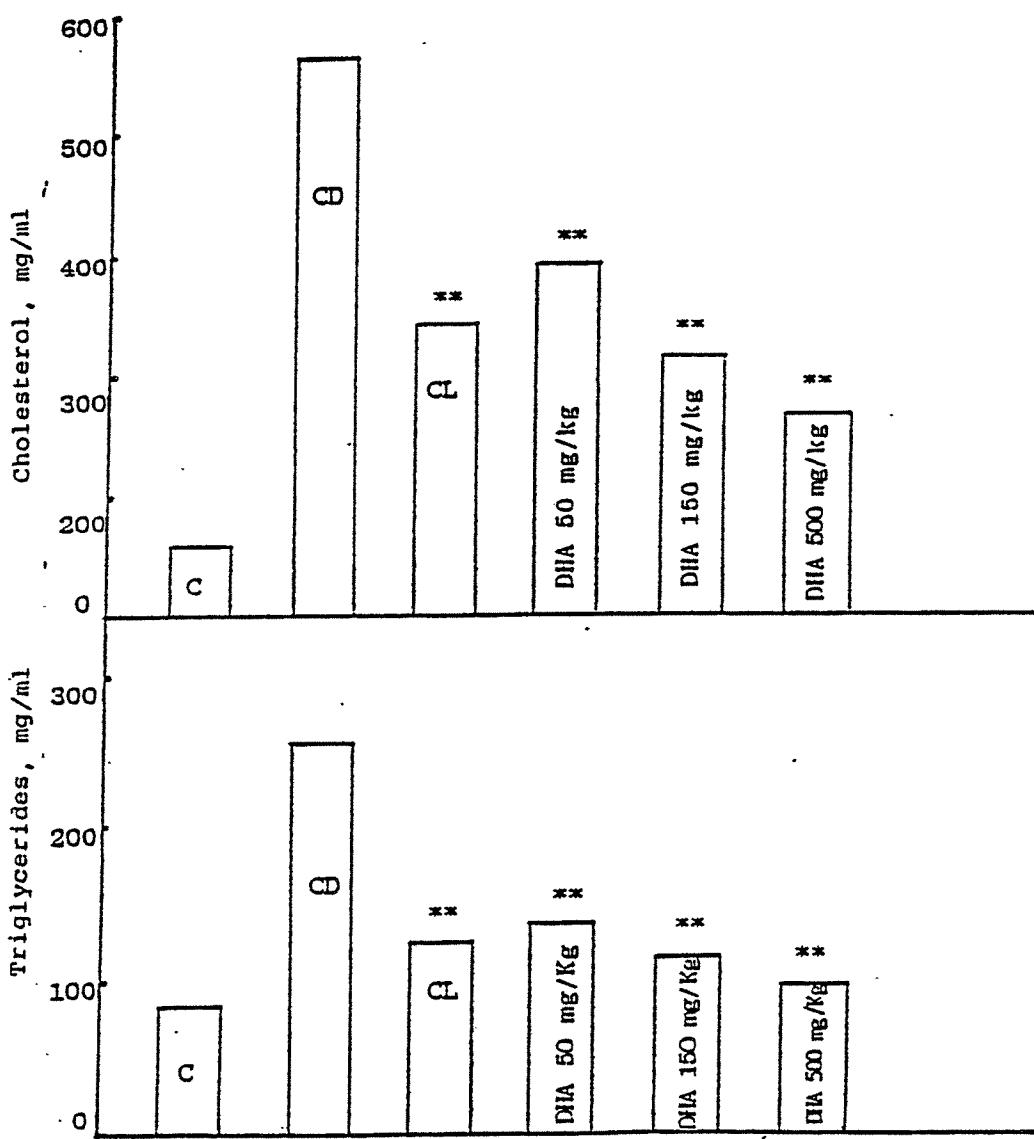


Figure 3

2/8

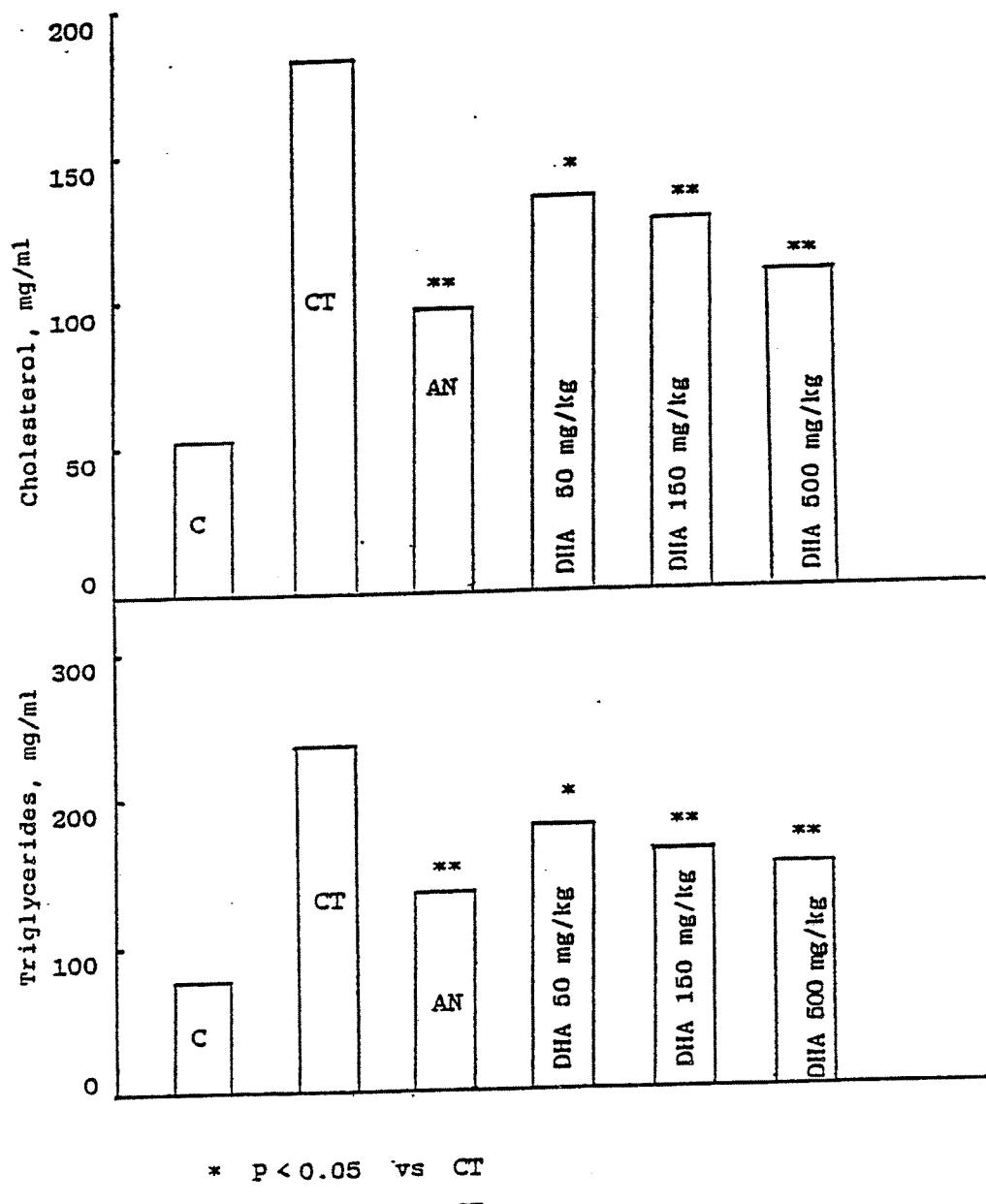
Figure 4 - Effect of the administration of highly purified (96%) DHA on rats affected by hypercholesterolemia and hypertriglyceridemia induced by Nath diet (preliminary data)



\*\* P < 0.01 vs CD

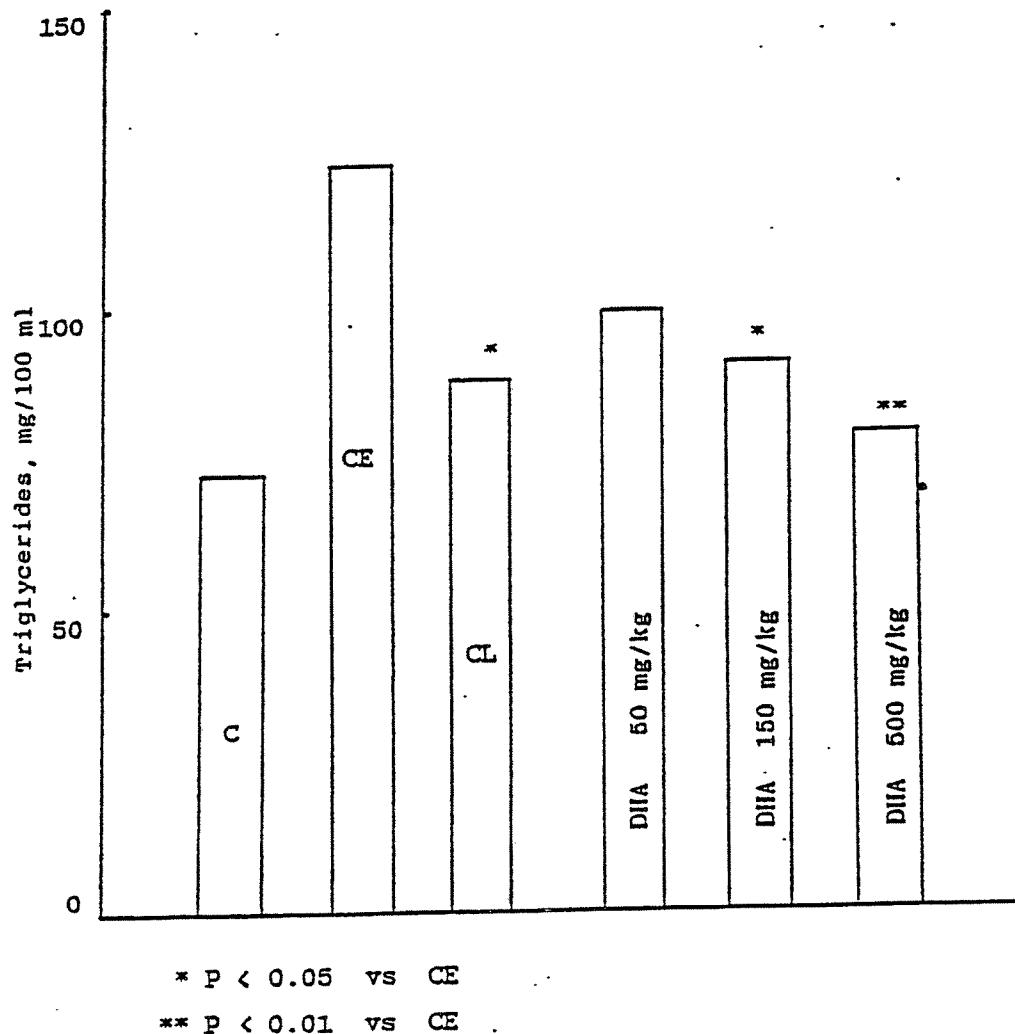
3/8

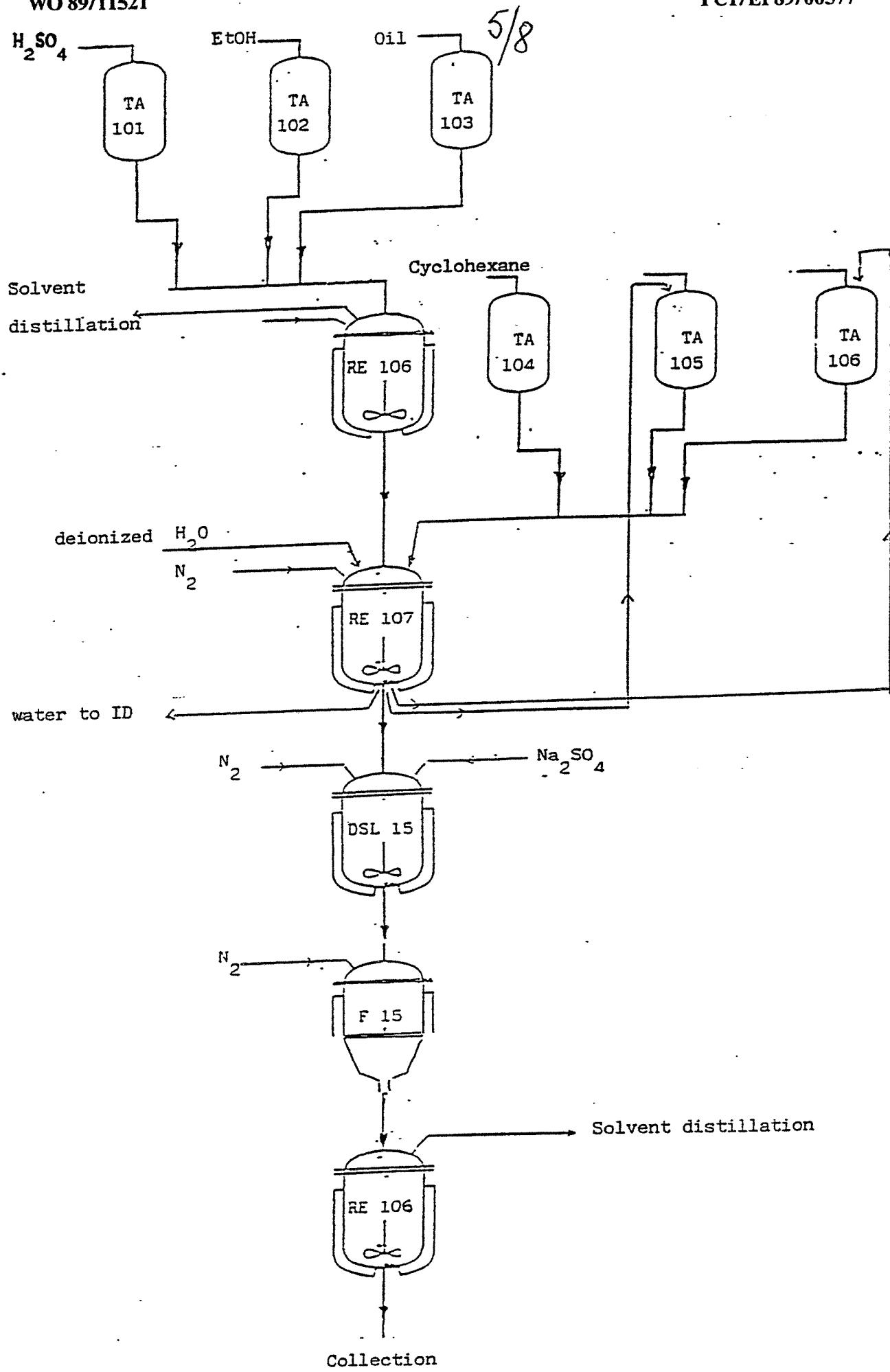
Figure 5 - Effect of the administration of highly purified (96%) DHA on rats affected by hypercholesterolemia and hypertriglyceridemia induced by WR 1339 (preliminary data)



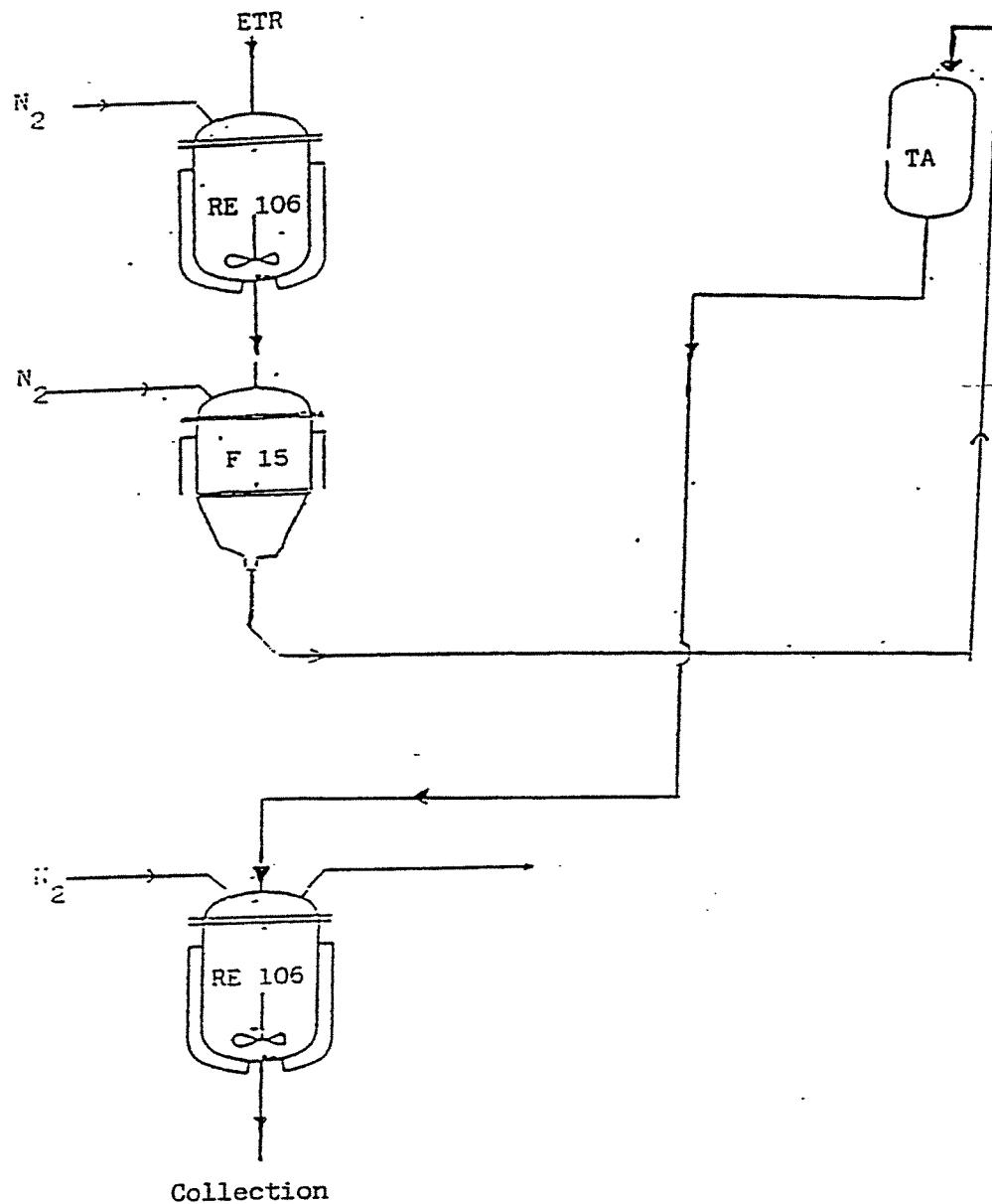
4/8

Figure 6 - Effect of the administration of highly purified (96%) DHA on rats affected by hypertriglyceridemia induced by ethanol (preliminary data)



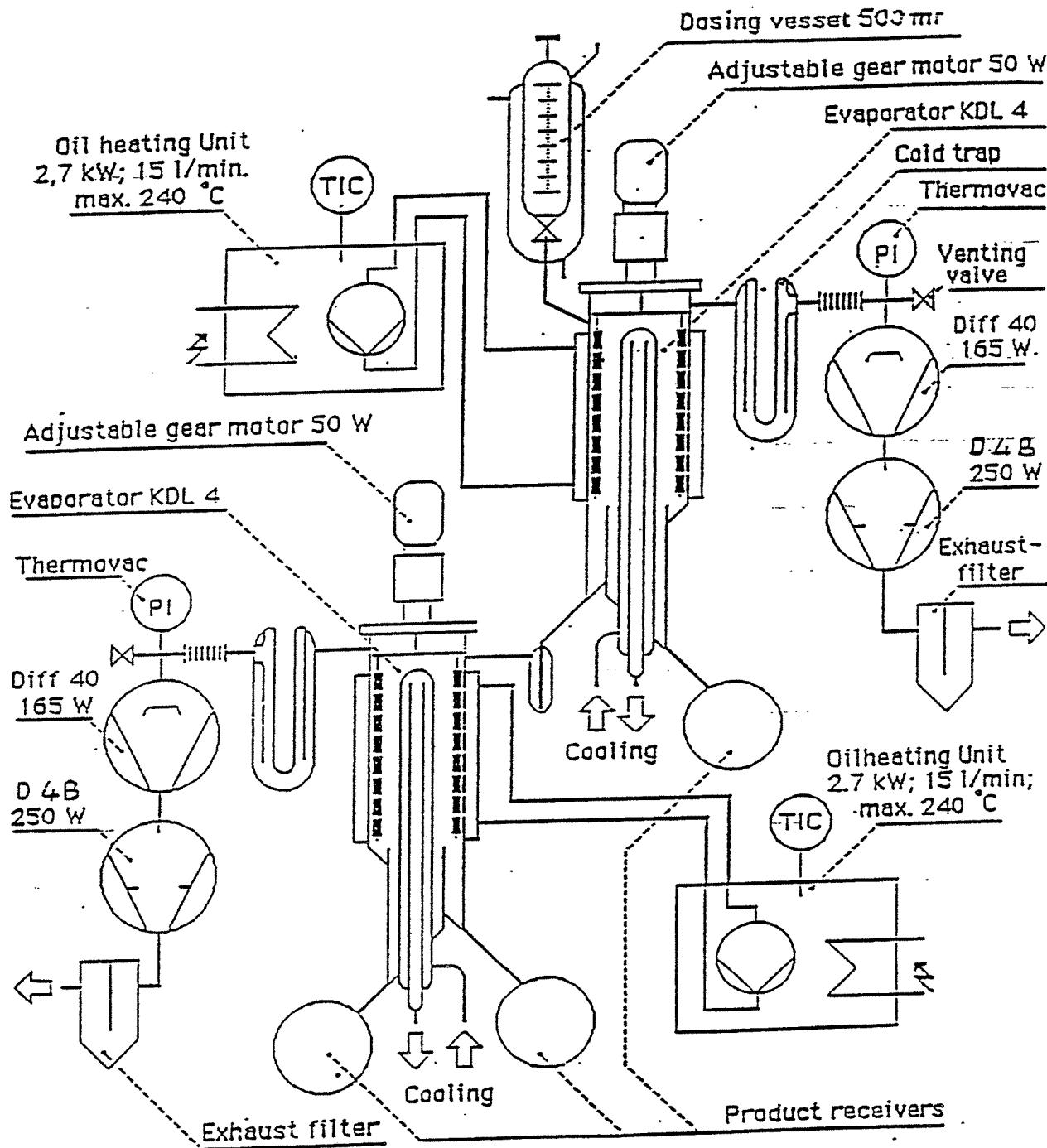


6/8

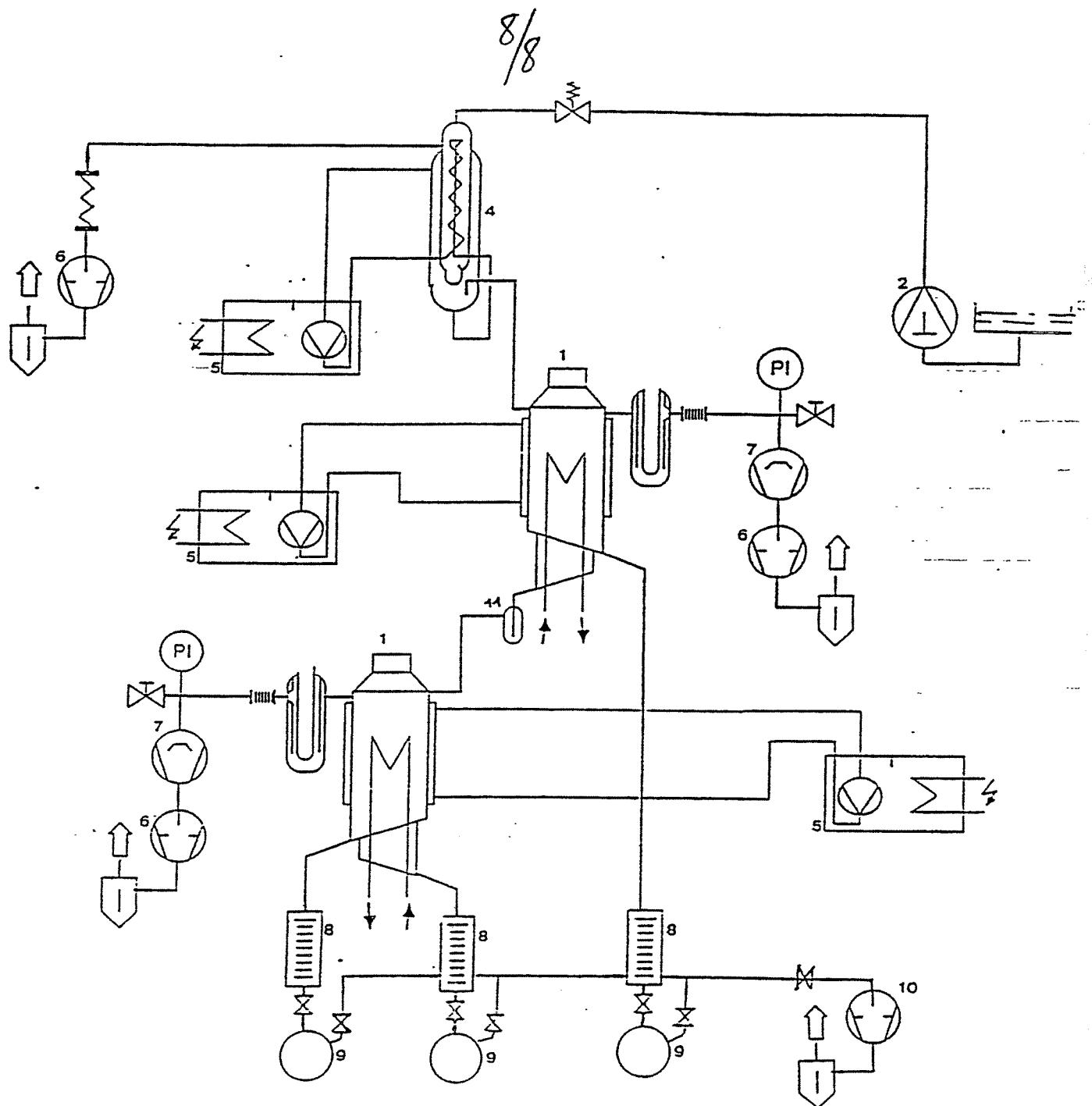


7/8

Schema n.53



SHORT PATH DISTILLATION UNIT  
DOUBLE STAGE

**Description:**

1. Evaporators	7. Diffusion pump
2. Metering pump	8. Graduated cylinders with venting valves
3. Dosing vessel	9. Product receivers
4. Degasser	10. Rotary pump D8B
5. Heating unit	11. Running trap from stage I to stage II
6. Rotary pump D4B	PI Thermovac

# INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 89/00577

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 11 C 1/10, 1/02, C 07 C 51/44, 57/03, A 61 K 31/20,  
A 23 L 1/30

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System	Classification Symbols
IPC <sup>4</sup>	C 11 C, C 07 C, A 61 K, A 23 L

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT\*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
P,X	EP, A, 0292846 (INNOVA DI RIDOLFI FLORA & C. S.a.S.) 30 November 1988, see claims 1,2; example; page 1, lines 43-49; page 2, lines 22-23,28-49 --	1-17
X	US, A, 3082228 (L.H. SUTHERLAND) 19 March 1963, see claims 1,3,4; column 1, line 15 - column 2, line 70; column 3, line 61 - column 4, line 63; examples 1-4 --	1-17
X	Derwent File Supplier WPI(L) 1988, AN - 88-145139 (21) Derwent Publications Ltd (London, GB) & JP, A, 63088159 (NIPPON OILS & FATS K.K.) 19 April 1988, see abstract --	1,4,5,6, 11,13,14
P,X	WO, A, 88/08444 (MTA SZEGEDI BIOLOGIAI KÖZPONTJA) 3 November 1988, see page 9, line 8; page 16, example 7 --	1,4,7,9, 14-16

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

23rd August 1989

Date of Mailing of this International Search Report

22.09.89

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

L. ROSSI

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	GB, A, 2090529 (NIPPON OIL AND FATS CO. LTD) 14 July 1982, see page 2, lines 13-21 (cited in the application)	1
A	see claims 1,3 --	4,5,11,14-17
Y	GB, A, 2152043 (THE NISSHIN OIL MILLS LTD (JAPAN)) 31 July 1985, see claims 1,3,10; page 2, lines 13-24; example 1	1
A	--	4,7,15,16
A	Derwent File Supplier WPI(L) 1984 AN - 84-198209 (32) Derwent Publications Ltd (London, GB) & JP, A, 59113099 (OISHI K) 29 June 1984, see abstract --	1,4,7,9, 15,16
A	US, A, 4554107 (MASAYASU TAKAO) 19 November 1985, see column 1, line 64 - column 2, line 7; column 4, lines 9-20,44-51; examples 1,2	1,4,7,9, 15,16
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 8900577  
SA 28892

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 15/09/89  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0292846	30-11-88	JP-A-	1070439	15-03-89
US-A- 3082228		None		
WO-A- 8608444		None		
GB-A- 2090529	14-07-82	JP-A-	57035512	26-02-82
		WO-A-	8200095	21-01-82
GB-A- 2152043	31-07-85	JP-A-	60133094	16-07-85
		DE-A,C	3446795	04-07-85
		US-A-	4615839	07-10-86
US-A- 4554107	19-11-85	JP-A-	60023493	06-02-85
		US-A-	4623488	18-11-86